VIA MEDICA

ORIGINAL ARTICLE

Vol. 61, No. 1, pp. 7–14

ISSN 0015–5659
www.fm.viamedica.pl

Cholinergic innervation and calretinin--immunoreactive neurones in the hippocampus during postnatal development of the rat brain

Beata Ludkiewicz, Sławomir Wójcik, Edyta Spodnik, Beata Domaradzka-Pytel, Janusz Moryś

Department of Anatomy and Neurobiology, Medical University of Gdańsk, Poland

[Received 4 January 2002; Accepted 28 January 2002]

Immunohistochemical study of the cholinergic innervation of the hippocampal calretinin-containing cells was conducted on 28 rat brains of postnatal ages: P0, P4, P7, P14, P21, P30 and P60. Sections with double immunostaining for vesicular acetylcholine transporter (VAChT; the marker of cholinergic cells, fibres and terminals) and calretinin were analysed using confocal laser-scanning microscope. Obtained data demonstrate that during development as well as in adult species calretinin-containing neurones in the rat hippocampus form sparse synaptic contact with VAChT-ir terminals. It seems probable that cholinergic innervation is not crucial for the functioning of CR-ir cells — probably they remain under the greater influence of a system other than the cholinergic system.

key words: hippocampus, development, calretinin, cholinergic system

INTRODUCTION

The hippocampus is involved in the control of cognitive and emotive behaviour, particularly in learning and memory [17, 19, 37]. Two main groups can be distinguished among hippocampal neurones: principal and non-principal cells. The former include pyramidal and granule cells, which are excitatory and convey information between the various hippocampal subfields [2, 9, 25]. Most non-principal cells are GABAergic and nonpyramidal; they inhibit activity of principal cells [6, 38]. The population of GABAergic nonpyramidal neurones is rather heterogeneous in their morphology, synaptic connections, content of neuroactive substances and calcium binding proteins (CaBPs): parvalbumin, calbindin D28k, and calretinin [25]. CaBPs play a crucial role in the control of the level of calcium in the cytoplasm, which is thought to be critical for proper development [3, 18], for instance, for neurite outgrowth [12], neuronal migration [20, 21], and expression of neurotransmitter receptors [34].

Hippocampal neurones appear to be the major target for cholinergic septohippocampal fibres arising from the medial septum/diagonal band complex [1, 24]. Their endings are localised on cells in all hippocampal cell layers [10, 11] and the cholinergic system seems to be essential for the function of this structure [4]. Cholinergic septohippocampal neurones are believed to influence memory and attention processing [5, 7]. Furthermore, in developmental processes the cholinergic system plays an important role in the proliferative processes and axon guidance [22, 23]. As a good marker of the cholinergic system two antibodies were used: anti-ChAT (against the enzyme synthesising acetylocholine) and anti-VAChT (against vesicular acetylocholine transporter). The latter presumably transports ACh into synaptic vesicles to regulate release upon stimulation [8, 27, 31]. Unlike ChAT,

Address for correspondence: Prof. Janusz Moryś, MD, PhD, Department of Anatomy and Neurobiology, Medical University of Gdańsk, ul. Dębinki 1, 80–211 Gdańsk, Poland, tel: +48 58 349 1401, fax: +48 58 349 1421, e-mail: jmorys@amg.gda.pl

VAChT protein is an integral membrane protein mainly localised in the synaptic vesicles. Since synaptic vesicles occur in higher concentrations in the nerve terminals, the anti-VAChT antibody should be a useful marker of cholinergic synapses as well as in fibres in all known cholinergic projection fields [29]. In resume, anti-ChAT serves as a better marker for cholinergic somata and fibres, whereas anti-VAChT — for cholinergic terminals.

Due to the distinct expression of pattern of CaBPs during development [18], in the present study we investigated developmental relationship between cholinergic innervation and the neurones containing calretinin (CR) in the hippocampus using immunohistochemical methods. For detection of cholinergic fibres and terminals, we used a specific antibody against VAChT [30].

MATERIAL AND METHODS

The material consisted of 28 Wistar rat brains of various postnatal ages: P0, P4, P7, P14, P21, P30 and P60. In each group four animals were studied. Care and treatment of the animals were in accordance with the guidelines for laboratory animals established by the National Institutes of Health as well as by the Local Ethical Committee of the Medical University of Gdańsk. All animals were deeply anaesthetised with lethal does of Nembutal (80 mg/kg of body weight), then transcardially perfused with 0.9% solution of NaCl with heparin, followed by 4% solution of paraformaldehyde in 0.1M phosphate buffer (pH 7.4; 50–250 ml). The brains were postfixed in 4% paraformaldehyde fixative for 3–4 hours, and then kept in 0.1 M phosphate buffer containing 10% sucrose (overnight at 4°C) and 30% sucrose (until sunk).

Coronal 40-μm-thick, serial sections of the brain were cut on JUNG 1800 cryostat (Leica, Germany). The free-floating sections were blocked for 1 hour with 3% NGS containing 0.4% Triton X-100 and then incubated for 48 hours in 4°C with the mixture of the goat anti-VAChT and rabbit anti-calretinin antibodies diluted in 3% NGS (Table 1). After multiple rinses in PBS, sections were incubated (2–3 hours, at room temperature) with the mixture of the appropriate secondary antibodies conjugated with the FITC or Cy3 (Table 1).

The specificity of staining was checked according to the procedure described by Wouterlood et al. [41].

The histological sections were studied under the MicroRadiance AR-2 (Bio-Rad, UK) confocal laser-scanning microscope equipped with an Argon laser producing dichromatic light at 488 and 514 nm. The 488-nm line of this laser was applied to excite the fluorescein isothiocyanate (FITC), using a dichroic beam splitter FT 505 and an emission long-pass filter LP 515. The 514-nm line of this laser was applied to excite Cy3, using an excitation filter 514 and an emission long-pass filter E570LP. For 3D reconstruction the image analysis program LaserSharp 2000 v. 2.0 (Bio-Rad; UK) was used.

Table 1. Specifications and dilutions of the primary and secondary antibodies

Antibodies	Manufacturers	Dilution
Goat anti-VAChT *(polyclonal)**	Chemicon	1:1000
Rabbit anti-calretinin *(polyclonal)**	Chemicon	1:1000
Cy™3-conjugated Donkey anti-Goat**	Jackson Immuno Research	1:800
FITC-conjugated Donkey anti-Rabbit**	Jackson Immuno Research	1:100

* primary antibodies, ** secondary antibodies

For subdivision of the hippocampal formation we applied procedures used by Sloviter [32] and Jiang et al. [18] (Fig. 1).

RESULTS

VAChT-immunoreactivity

In P0 VAChT-immunoreactive (-ir) puncta were present in both sectors of hippocampus proper (CA1 and CA3) as well as in the dentate gyrus (DG). During the first postnatal week the amount of immunopositive puncta clearly increased and for the first time VAChT-ir fibres became visible (since P4). Network of immunoreactive puncta and processes with varicosities appeared on P14 and since that time there were no major changes in the distribution and morphology of VAChT immunoreactivity. More intense immunoreactivity was observed in the vicinity of principal cell layers (pyramidal and granular; Fig. 2, 3, 4). VAChT-ir elements frequently formed "basket" structures that often surrounded the CR-immunonegative cells (Fig. 2, 3, 4). During the whole studied period of postnatal life VAChT-positive neurones were observed neither in any hippocampal sector nor in DG.

CR-immunoreactivity

In general, distribution and immunoreactivity of calretinin containing neurones during postnatal development of rat hippocampus were similar to that previously described [18].

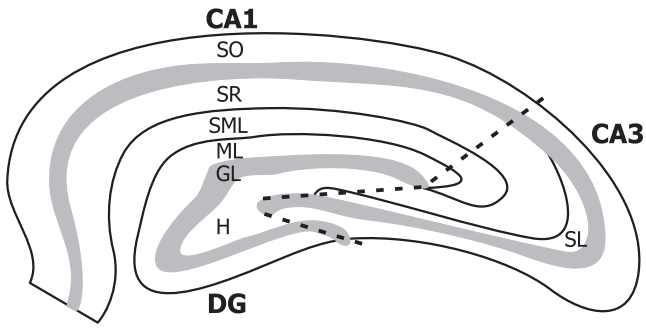

Figure 1. Diagram of the rat hippocampus. Dotted lines separate sector CA1 from CA3 and CA3 from dentate gyrus (DG); SO — stratum oriens, SP — stratum pyramidale, SR — stratum radiatum, SLM — stratum lacunosum-moleculare, ML — molecular layer of dentate gyrus, GL — granule cell layer, H — hilus of dentate gyrus, SL — stratum lucidum (from Jiang and Swann [18]).

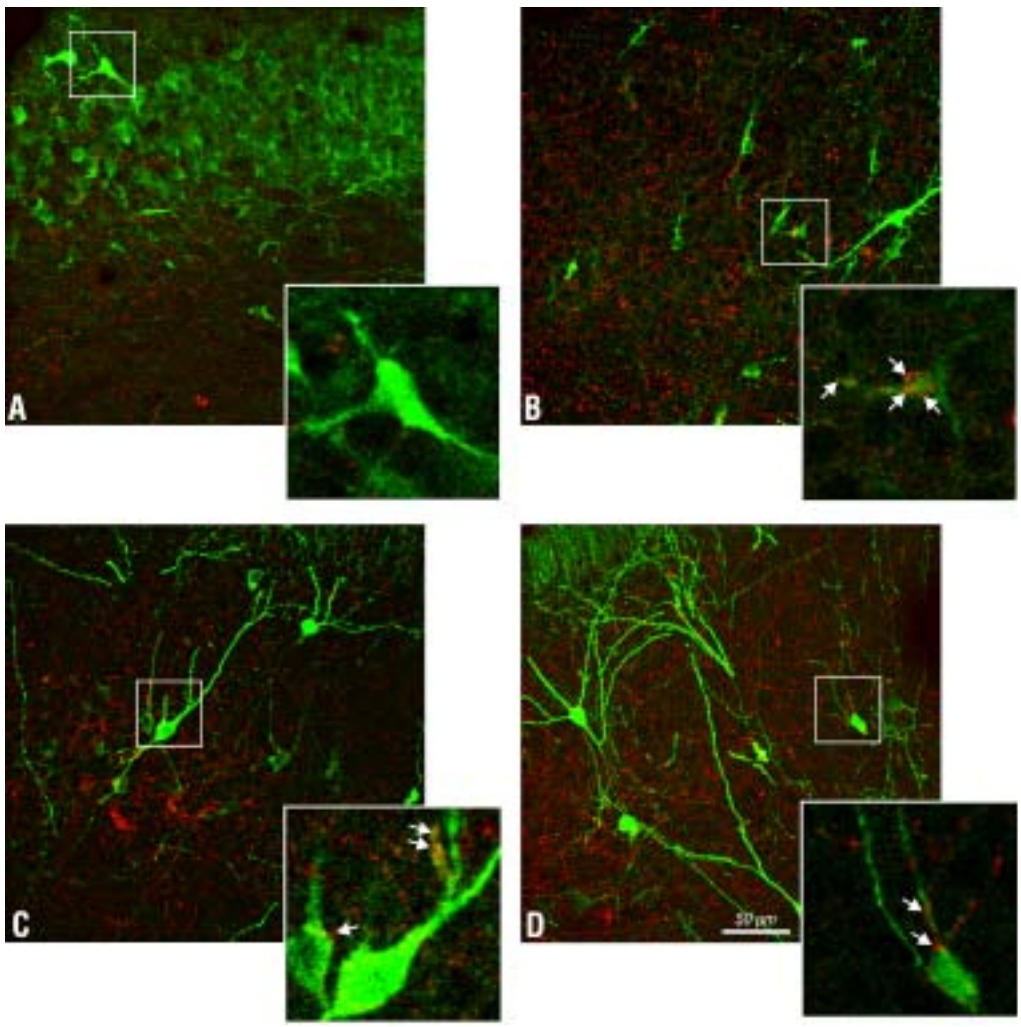

Figure 2. CR-positive cells (green) and VAChT-positive fibres and terminals (red) in CA1 sector of hippocampus proper at stages: P4 (**A**), P7 (**B**) P14 (**C**), P21 (**D**). White arrows indicate VAChT-positive puncta forming synaptic contact with CR-ir neurones; P — postnatal day.

On P0 CR-immunoreactive elements were observed in the hippocampus proper (mainly in CA3 sector) and in the dentate gyrus (Figs. 3A, 4A). In the hippocampus proper CR-ir cells appeared to be located in the pyramidal cell layer, stratum radiatum and stratum oriens (Fig. 3A); in the dentate gyrus they were ob-

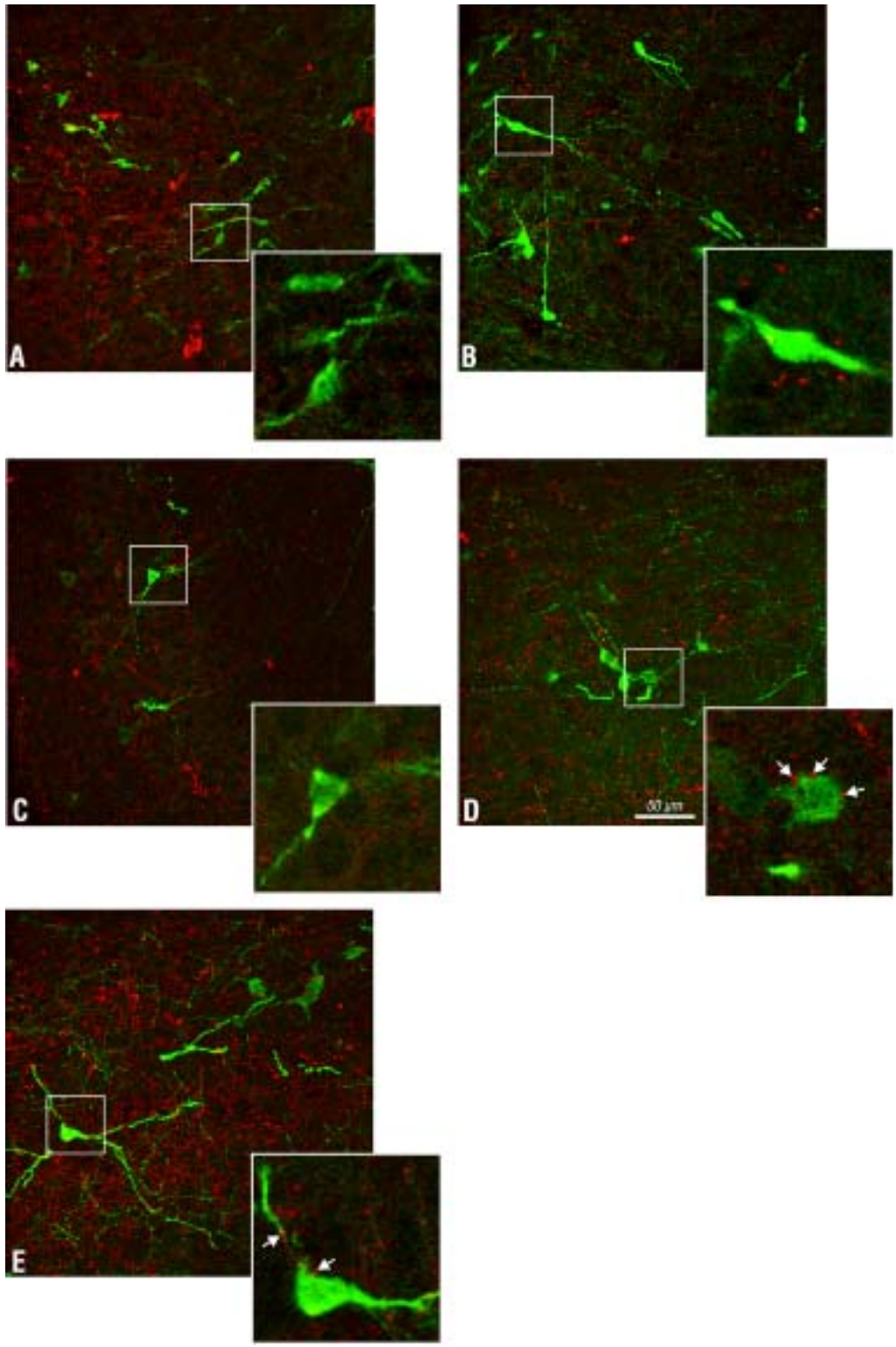

Figure 3. CR-positive cells (green) and VAChT-positive fibres and terminals (red) in CA3 sector of hippocampus proper at stages: P0 (**A**), P4 (**B**) P7 (**C**), P14 (**D**), P21 (**E**). White arrows indicate VAChT-positive puncta forming synaptic contact with CR-ir neurones; P — postnatal day.

served mainly in the hilus (Fig. 4A). Perikarya of the CR-ir neurones were of various shapes (often fusiform). They also had sparse short processes (Figs. 3A, 4A).

On P4 the number of CR-ir cells increased, particularly in CA1 sector of the hippocampus proper and somewhat in CA3 sector. At this time a new

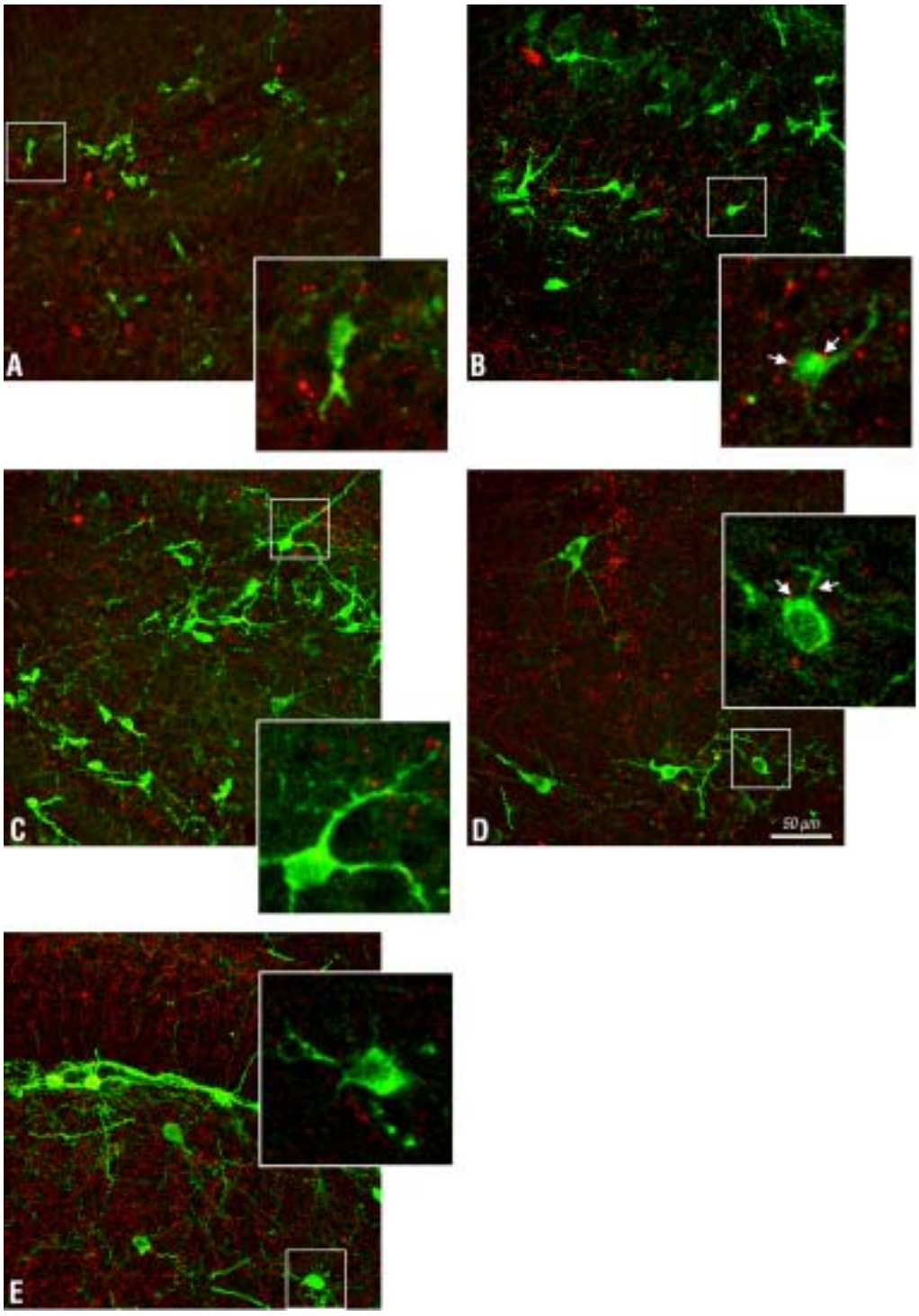

Figure 4. CR-positive cells (green) and VAChT-positive fibres and terminals (red) in dentate gyrus at stages: P0 (**A**), P4 (**B**) P7 (**C**), P14 (**D**), P21 (**E**); P — postnatal day. White arrows indicate VAChT-positive puncta forming synaptic contact with CR-ir neurones.

population of small CR-ir neurones of various shapes was observed between the stratum lacunosum-moleculare of CA1 sector and the molecular layer of dentate gyrus. On P7 these cells were still present although they were fewer in number; they disappeared at the end of the second postnatal life.

Since P21, the level and distribution of CR immunoreactivity did not change significantly. In sector CA1 CR-ir cell bodies were observed mainly in or near the

pyramidal layer; their dendrites, often displayed beaded or varicose features, usually branched in the stratum oriens and stratum radiatum (Fig. 2D). CR-ir cells were localised in sector CA3 mainly in pyramidal layer (Fig. 3E); their dendrites branched locally. In the dentate gyrus, numerous CR-ir neurones were located horizontally just beneath the granule cell layer; immunoreactive cells were also observed inside the hilus — their processes branched locally (Fig. 4E).

Cholinergic innervation of calretinin positive cells

3D reconstruction of double immunostained sections has shown that until P4 VAChT-positive puncta only sporadically formed synaptic contact with CR-ir neurones (Figs. 3A, 4A). Analysing the relations of CR-ir cells with VAChT-positive terminals since P7, two unequal subpopulations of CR-ir neurones seem to be present: intensively stained neurones devoid of synaptic contact with cholinergic terminals (Figs. 3C, 4C, E) and somewhat lightly stained neurones forming sparse synaptic contacts with cholinergic terminals. The former sub-population predominated. VAChT-positive endings were observed mainly on CR-ir somata, rarely on processes (Figs. 2B–D, 3D, E, 4D). Cells of the above-mentioned sub-populations were randomly distributed and they were not limited to any hippocampal region or layer.

DISCUSSION

The distribution and immunoreactivity of CR-ir cells in the hippocampus during maturation as well as in the adult animals observed in present study were similar to those previously described [18].

According to Gulyas at al. [15] two major types of CR-ir GABAergic neurones have been distinguished in the rat hippocampus, i.e. spiny and aspiny. The latter form symmetrical synaptic contacts exclusively on GABAergic dendrites. The unique connectivity of these cells may enable them to play a crucial role in generation of synchronous, rhythmic hippocampal activity by controlling other interneurones terminating on a different dendritic and somatic compartment of principal cells [9, 14, 15].

The spiny CR-ir cells form a distinct population of inhibitory non-pyramidal cells of hilus and CA3 sector; they influence the activity of dentate granular cells, hilar and CA3 pyramidal neurones [18, 25] and may have a specialised function such as synchronising the activity of CA3 pyramidal cells [16, 26]. Spiny CR-ir cells are innervating mainly by mossy fibres (glutamatergic afferents originating exclusively from granule cells [16, 36]). Moreover these neurones presumably receive their synaptic input through the perforant path [39, 39, 40] and may exert feed-forward inhibition on the dendrites of principal cells that may also receive input from the perforant pathway. Therefore, this neuronal population may influence the susceptibility of dendrites of hippocampal principal neurones (both granule and pyramidal) to neocortical input [26]. Additionally, the calretinin is expressed in transitory sub-population, such as Cajal-Retzius cells. This suggests that calretinin could play an important role in the developmental processes that take place over a limited period of time [18]. According to Soriano et al. [33], hippocampal Cajal-Retzius cells may play a crucial role in the guidance of axons of the perforant path.

According to our results, VAChT-positive puncta (considered to be axon terminals, although they may be transversely cut fibres [13]) were observed in the hippocampus already at early postnatal period, initially as puncta, later as puncta and fine varicose processes. These data differ from those of Gould et al., [13] who described that cholinergic fibres deriving from the medial septum/diagonal band complex were not detectable in the hippocampus before P10 [13]. The discrepancy between the results of Gould et al. [13] and our findings seems to be due to the usage of different antibodies as markers of cholinergic fibres: Gould et al. [13] utilised an antibody against the enzyme synthesising acetylocholine (ChAT), whereas in our study an antibody against vesicular acetylcholine transporter (VAChT) was applied.

The results of the present study suggest that the cholinergic innervation of calretinin-immunopositive hippocampal neurones both during development as well as in adult are rather scanty. Even though on P0 both CR-ir neurones as well as VAChT immunoreactive elements were visible in hippocampus proper and in dentate gyrus until P4 they formed synaptic contact only sporadically. VAChT-positive endings were observed mainly on CR-ir somata, rarely on processes. Due to the different physiological properties of these types of endings (regulation of action potential firing by somatic synapses and influence efficacy of afferents by dendritic synapses [28]), perikarial contact may exert a more rapid and potent effect on the cell than dendritic inputs do [7, 35]. Therefore, despite the low number of cholinergic endings on the CR-ir neurones, their influence on the cell may be fairly significant. However, the sub-population of cells forming sparse synaptic contact is smaller - so the total effect of cholinergic afferents upon CR-it neurones seems to be small.

The present findings clearly demonstrate that both during development as well as in adult stage, the calretinin-containing neurones in the rat hippocampus form only sparse synaptic contact with VAChT-ir terminals. They suggest that cholinergic innervation should not be crucial for the functioning of CR-ir cells - probably they remain under the greater influence of a system other than the cholinergic system.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs Aleksandra Arceusz MA, for copy-editing the manuscript and Ms Sylwia Scisłowska for the preparation of illustrations. This paper is supported by funds from the Committee of Scientific Research, CSR Grant ST-11.

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